Journal of Applied Microbiology 1997, 83, 678–684

# Specific peptides of casein pancreatic digestion enhance the production of tetanus toxin

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6009/11/96: received 28 November 1996, revised 19 March 1997 and accepted 2 April 1997

Z. PORFÍRIO, S.M. PRADO, M.D.C. VANCETTO, F. FRATELLI, E.W. ALVES, I. RAW, B.L. FERNANDES, A.C.M. CAMARGO AND I. LEBRUN. 1997. Casein pancreatic digest is the basic bacterial growth medium used for diphtheria, botulinum and tetanus toxin vaccine production. It is known that the variation in the peptide content of the casein digest directly affects final toxin yields. In this study, the identification and sequences of eight peptides, four to eight amino acids in length, of casein pancreatic digestion, which seem to be involved in the enhancement of tetanus toxin production, are described. They all contain one or two residues of proline/molecule and a predominance of hydrophobic amino acid residues. The most active peptides show a general structure of Pro-aromatic-Pro, and this pattern resembled the motif displayed by bradykinin-potentiating peptides found in snake venoms. By analogy with the mechanism of bradykinin potentiation through inhibition of the proteolytic degradation of bradykinin, it is suggested that the six peptides identified here could protect the tetanus toxin from proteolysis, once secreted by the bacteria.

## INTRODUCTION

Until recently, it was thought that milk casein was only particularly important as a source of essential amino acids for growth and body development, and as a calcium carrier in mammals (for review see McKenzie 1967). Surprisingly, however, partial proteolysis of casein may generate biologically-active peptides which display opiate, cardiovascular or respiratory activities, or which may even act as immunomodulators (Brantl *et al.* 1979; Maruyama and Suzuki 1982; Hedner and Hedner *et al.* 1987). Some of these peptides are peptidase inhibitors, such as Angiotensin converting enzyme and endooligopeptidase A (Maruyama *et al.* 1987; Lebrun *et al.* 1995); they may also act as agonists on specific opiate receptors, or even interfere with second messenger pathways (Zioudrou 1979; Maruyama *et al.* 1985, 1987; Lebrun *et al.* 1995).

Milk casein has been used as an essential nutritional supplement in bacterial growth media, particularly those used in the production of tetanus, diphtheria and botulinum vaccines. In addition, small peptides present in the neutral fraction of the casein hydrolysate media were found to increase tetanus toxin yields (Mueller and Miller 1954).

In this report, the isolation of several oligopeptides from a commercial dehydrated preparation of casein (Pancreatic Digest of Casein, NZCase TT, Sheffield Farms & Co., NY, USA), and their primary structures, are described. These peptides were shown to stimulate both the growth of *Clostridium tetani* and the production of tetanus toxin, which is a 150 000 Da protein composed of two chains (100 000 Da and 50 000 Da) held together by a disulphide bond (Simpson 1986).

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## MATERIAL AND METHODS

### Compounds

The casein pancreatic digest (CPD; NZCase TT) was obtained from Sheffield Farms & Co., NY, USA.

### Basic medium for bacterial growth

The culture medium was described by Mueller and Miller (1954) and modified by Lathan (1962). Components of the media are: pancreatic digest of casein 25 g $l^{-1}$ (2·5%); $KH_2PO_4$ 1·01 g $l^{-1}$, $CaCl_2$ 0·72 g $l^{-1}$; active charcoal 0·36 g $l^{-1}$; glucose 8·0 g $l^{-1}$; NaCl 2·5 g $l^{-1}$; $MgSO_4$ 0·1 g $l^{-1}$; Biotin 1·6 μg $l^{-1}$; Cyanocobalamin 0·5 μg $l^{-1}$; Tiamin 0·25 mg $l^{-1}$; nicotinic acid 0·25 mg $l^{-1}$; Uracil 1·25 mg $l^{-1}$; $FeCl_3$ 32 mg $l^{-1}$; Cystin 125 mg $l^{-1}$. The final medium was distributed in assay tubes and sterilized at 120 °C for 20 min. When required, fractions of CPD obtained after chromatography were added to the basic medium containing 1% CPD until a concentration of 2·5% (w/v) was reached.

### Thioglycollate medium (Brewer)

Components of the media are: pancreatic digest of casein 15 g $l^{-1}$; NaCl 2·5 g $l^{-1}$; glucose 5·0 g $l^{-1}$; yeast extract 5·0 g $l^{-1}$; agar 750 mg $l^{-1}$; Cystin 750 mg $l^{-1}$; sodium thioglycollate 500 mg $l^{-1}$; methylene blue (0·02% solution) 10 ml $l^{-1}$. The final medium was distributed in assay tubes and sterilized at 120 °C for 20 min.

### Chr matographic columns and solvents

Dowex-50 W $H^+$ ionic exchange columns were from Sigma (St Louis, MO, USA) and all eluents were from Merck (Darmstadt, Germany; PA grade). HPLC solvents (Lichrosolv or HPLC grade) were from Merck or Omnisolv. TSK ODS 120 T columns were from Pharmacia (Uppsala, Sweden), and μBondapack from Millipore (Bedford, USA).

### Animals

Male and female Swiss albino mice (18–22 g) were from the animal house of the Butantan Institute.

### Bacterial strain

*Clostridium tetani* Harvard strain was supplied by the Anaerobic Vaccine Section of the Butantan Institute. Stock cultures were grown in thioglycollate media at 36·5 °C for 24 h, distributed in tubes (2 ml each), and frozen at −20 °C.

### Tetanus toxin production

Clostridium tetani *growth.* Culture media in test tubes (15 ml media) or in beakers (2 l media) were inoculated with 0·3 or 5·0 ml, respectively, of a *Cl. tetani* preculture grown in thioglycollate medium for at least 24 h to reach a density of 0·6 $O.D._{560\,nm}$. Cultures were always grown at 36·5 °C for 7 d, except for toxin production optimization. In this case, 5·0 ml samples were collected from the 2 l culture at 0, 3, 6, 9, 12, 24, 36, 48, 60, 84, 108, 132 and 156 h and the Lf $ml^{-1}$ values determined (Fig. 1a).

For casein content optimization, 15 ml of medium with 0·5% stepwise increases of NZCase from 0·5 to 5% were used and the Lf $ml^{-1}$ determined (Fig. 1b).

*Flocculation test (Lf).* The method for obtaining tetanus toxin titres corresponding to the tetanus toxin yield was described by Ramon (1922). The values obtained were compared to a standard tetanus antitoxin scale, derived from a 100 Lf $ml^{-1}$ sample, using a 10 tube scale with different volumes of the standard antitoxin and 1·0 ml of the bacterial supernatant fluid obtained by centrifugation (2500 *g*, 20 min). The Lf value is taken as that from the first tube in which flocculation occurs. Tests were run at 45 °C.

*Minimum Lethal Dose (MLD).* Minimum Lethal Dose was calculated to assure equipotentiality of the toxin produced by *Cl. tetani* grown in different media. It was determined using Swiss albino mice (18–22 g body weight) of either sex. Samples of the tetanus toxin produced were diluted in physiological solution, buffered with 1% peptone, pH 7·2, and

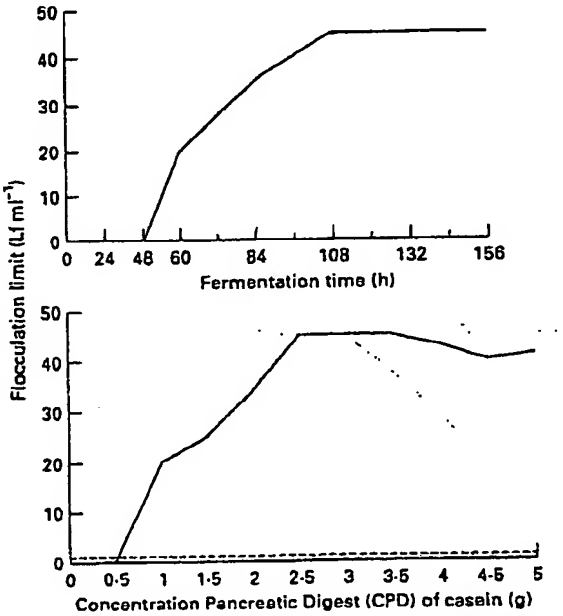



Fig.1 (a) Tetanus toxin production profile during 156 h using Lathan's media. (b) Effect of different concentrations of CPD in tetanus toxin production. Lf $ml^{-1}$ values were obtained as the lowest antitoxin dilution leading to flocculation with tetanus toxin

1·0 ml of the final solution was injected subcutaneously into each mouse. Ten mice were used and the MLD was the highest dilution of the toxin capable of producing tetanus symptoms and death of all mice in 96 h.

### Chromatography

*Dowex-50 W $H^+$ exchanger column.* A column (550 × 40 mm) filled with Dowex 50 W, 50–100 mesh, was repeatedly washed with 2 N NaOH, water, 2 N HCl, and finally equilibrated with 0·02 M $Na_2HPO_4$, pH 2·0. One litre of 10% pancreatic digest of casein solution, previously filtered and acidified with 30 ml concentrated HCl, pH 2·0, was applied, and the column eluted with: 3000 ml of 0·02 M $Na_2HPO_4$, pH 2·0 (acid fraction); 3000 ml of 0·02 M $Na_2HPO_4$, pH 7·0 (neutral fraction); and 2000 ml of 1 N $NH_4OH$ (basic fraction).

HPLC was performed in a Merck-Hitachi (Darmstadt, Germany) L-600 Gradient System using a semi-preparative reversed phase μBondapack C18 column (7·6 × 250 mm) with a linear gradient from 0 to 35% of acetonitrile in trifluoroacetic acid (0·1%) in 20 min, a flow rate of 2·5 ml $min^{-1}$, and monitored at 235 nm, or a reversed phase Beckman analytical column (4·6 × 250 mm) with a linear gradient from 0 to 50% of acetonitrile in trifluoroacetic acid (0·1%) in 20 min with a flow rate of 2·0 ml $min^{-1}$ and monitored at 214 nm.

*Amino acid sequence determination.* The amino acid sequence of the peptides was determined in an automatic sequenator using Edman's degradation method (1950) in a Shimadzu (Tokyo, Japan) PPSQ-10 and the PTH amino acids were identified in a C18 column. The resulting sequences were compared and alignments made using the BLAST-NCBI algorithm (Altshul *et al.* 1990).

### Statistical analysis

Statistical analyses of the data obtained in the different experiments were performed with the Minitab data analysis software from MINITAB Inc. for IBM PC computers. Tests used were Student *t*-test or one way ANOVA test, as required, using a 0·05 significance level.

## RESULTS

### Standardization of tetanus toxin production and CPD concentration

The *Cl. tetani* toxin production profile was analysed to obtain the optimum incubation time for the bacteria. The best growth time obtained was 108 h when a plateau was reached (Fig. 1a). The best CPD concentration was 2·5%, whereas 1% was the minimum for growth and toxin production (Fig. 1b).

Purification of the CPD was started with a Dowex-50 W $H^+$ ionic exchange chromatography column eluted in three steps with different pH buffers, 0·02 M $Na_2HPO_4$, pH 2·0 resulting in the acid fraction, 0·02 M $Na_2HPO_4$, pH 7·0 resulting in the neutral fraction, and 1 N $NH_4OH$, resulting in the basic fraction.

### Purification of peptide fractions and their effects on tetanus toxin production

Since the individual fractions do not allow *Cl. tetani* growth (Mueller and Miller 1956), the ability of combined fractions to induce tetanus toxin production was tested using the same concentrations of fractions present in the original media (acid 1%, basic 0·5% and neutral 1%). The results, presented in Fig. 2 showed that the media containing the neutral fraction made tetanus toxin production possible, yielding similar amounts as when the original medium containing 2·5% CPD was used. Similar results were obtained after addition of the peptide Gly His (Lathan 1962).

The neutral fraction obtained from the Dowex-50 W $H^+$ exchange chromatography column was purified by means of a semi-preparative HPLC column resulting in 10 main peaks (I to X) containing the peptide fractions (Fig. 3). Each peptide fraction was tested for its ability to stimulate tetanus toxin

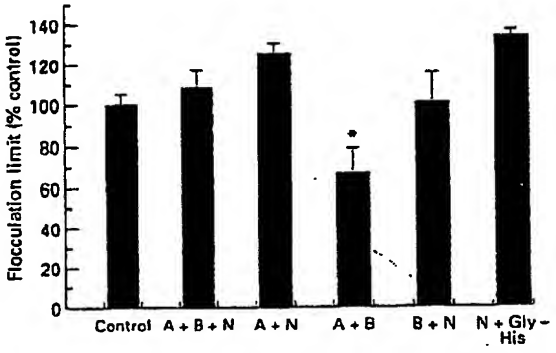
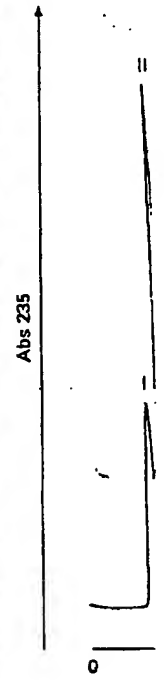



**Fig. 2** Effects of different combinations of CPD acid, basic and neutral fractions after Dowex 50 W $H^+$ chromatography on the production of tetanus toxin. The mean values of three assays are shown in percentage of control ± S.D. Mean values ranged from 40 to 80 Lf $ml^{-1}$. Significant differences, * $P < 0·05$ (Student *t*-test and one way ANOVA test) when compared to control. Fermentation time was 7 d. CPD fractions were used at the following concentrations: acid fraction (A) 1%; basic fraction (B) 0·5% and neutral fraction (N) 1%; dipeptide glycil-L-histidine (50 μg $ml^{-1}$); and control (CPD) 2·5%. Lf $ml^{-1}$ values were obtained as the lowest antitoxin dilution leading to flocculation with tetanus toxin



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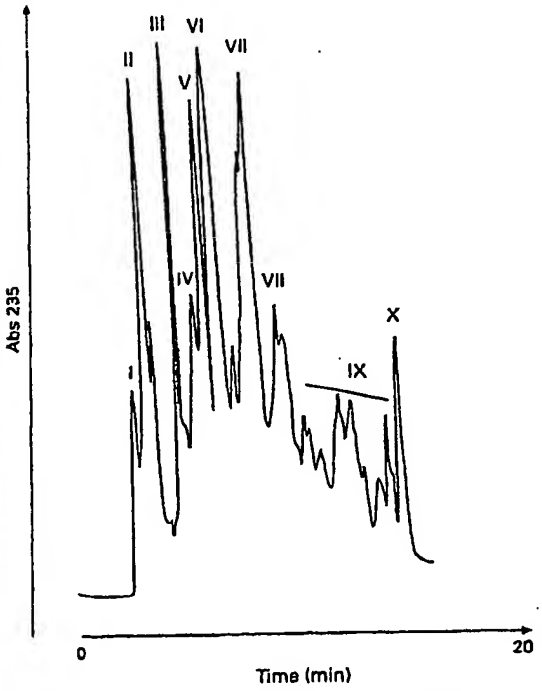



Fig. 3 Purification of active peptide fraction from CPD neutral fraction. Peptide fractions (peaks) derived from neutral fraction by HPLC chromatography were purified by means of a μBondapak C18 column, reversed phase, semi-preparative. A sample was applied to the column and eluted with a 0–35% linear gradient of acetonitrile in 0·1% trifluoroacetic acid (TFA) in water, for 20 min, with a flow rate of 2·5 ml $min^{-1}$ monitored at 235 nm. Ten main peaks were isolated, collected and then lyophilized

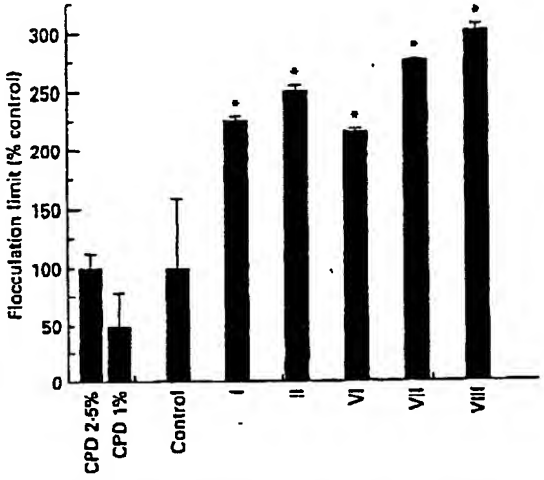



Fig. 4 Effects of different peptide components of the neutral fraction purified by HPLC chromatography on tetanus toxin production. The mean values of three assays are shown in percentage of control ± S.D. Mean values ranged from 20 to 60 Lf $ml^{-1}$. Significant differences * $P < 0.05$ (Student $t$-test and one way ANOVA test) when compared to control. Fermentation time was 7 d. CPD peptide fractions III, IV, IX and X did not flocculate. Lf $ml^{-1}$ values were obtained as the lowest antitoxin dilution leading to flocculation with tetanus toxin

production. The addition of fractions I, II, VI, VII and VIII to the basic growth medium resulted in an increase of 125, 150, 115, 175 and 200% of the control (CPD 1%), respectively (Fig. 4). Other fractions did not show significant changes (V), nor did they allow toxin flocculation (fractions III, IV, IX and X, data not shown).

### Purification, isolation and sequence of peptides and their effect on tetanus toxin production

The active peptide fractions VI, VII and VIII were repurified by means of an analytical HPLC column, and each peak was isolated (Fig. 5) and tested for its ability to stimulate tetanus toxin production (Fig. 6). Peptide fractions I, II and V were not further purified because of their high content of hydrophobic components, including hydrophobic amino acids and small peptides (di- and tripeptides) (data not shown). Each active peptide isolated from fractions VI, VII and VIII was sequenced, revealing peptides of four-eight residues with a predominance of hydrophobic amino acids and one or two prolines per molecule (Table 1). Ile-Pro-Ile-Gln-Tyr-Val (VI 1), Ala-Val-Pro-Tyr-Pro-Gln(VII d1) + Asp-Met-Pro-Ile (VII d2) were the most active peptides in their fractions (Fig. 6), reaching 165% and 150% above the control, respectively. Fractions VII b, VIII c, and VIII d also increased toxin production significantly, reaching 100% above the control. The sequences obtained revealed the $\beta$-casein chain to be the main source of active peptides.

## DISCUSSION

The influence of the culture media on *Cl. tetani* growth and toxin production was originally described by Mueller and Miller (1947, 1948, 1954, 1955, 1956). In these studies, *Clostridium* was grown in a complex medium containing casein hydrolysate and beef heart infusion. Later, Lathan (1962) showed that the heart infusion was not necessary since its removal did not affect the toxin yield. These authors claimed that homogeneity of the CPD constituents was important for bacterial growth and mainly for reproducible yields of toxin. This was confirmed by Stainer (1973) who observed sig-

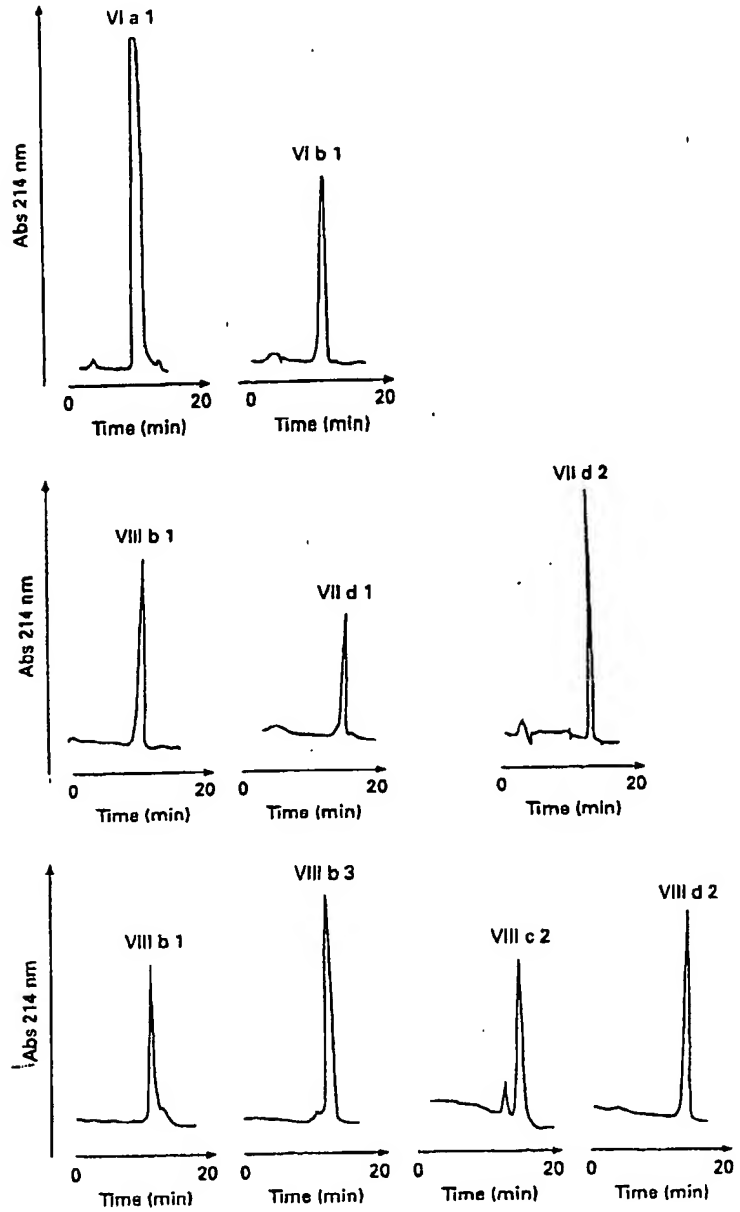
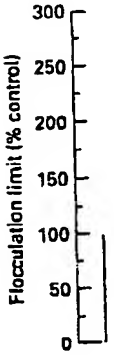



**Fig. 5** The VI[th] peak was eluted with three concentrations of B (isocratic steps of B) first step, 10 min in TFA, 2nd and 3rd steps with 5% and 10% of acetonitrile in TFA, with an elution time of 25 and 15 min, respectively, in a Resource RPC 3 ml semiprep reversed phase column with 2·5 ml $min^{-1}$ flow rate and monitored at 235 nm (not shown). The main peaks were collected and rechromatographed in an analytical TSK-ODS 120 T column (4·6 × 250 mm) with 2·5 $ml.min^{-1}$ flow rate and monitored at 214 nm. The isolated peptides were used for assays and sequence determinations. The same method was used for the VII[th] and the VIII[th] peak peptide isolation

nificant differences in Lf $ml^{-1}$ values using different batches of NZCase. These variations in the CPD composition represent a serious problem when they are reflected in vaccine yield.

The complexity of NZCase peptide content after pancreatic digestion was confirmed when compared with CPD medium obtained by digestion of crude casein with trypsin and chymotrypsin. This medium component allowed growth of *Cl. tetani* but did not stimulate high yields of tetanus toxin (data not shown). This suggested that these two enzymes were not the only ones responsible for the production of the active peptides present in the original media.



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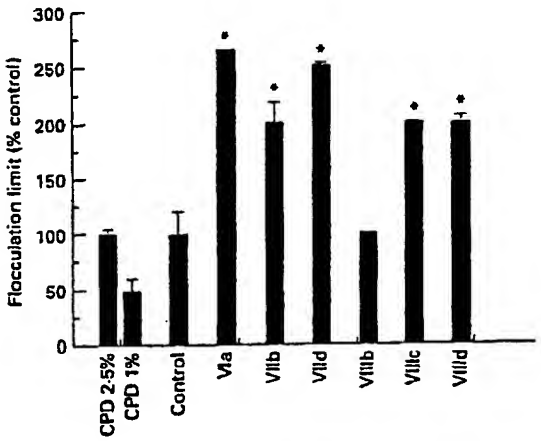


**Fig. 6** Effect of individual peptides isolated from peptide fractions VI, VII and VIII after HPLC repurification chromatography. The mean values of three assays are shown in percentage of control ± S.D. Mean values ranged from 20 to 53 Lf $ml^{-1}$. Significant differences * $P < 0{\cdot}05$ (Student *t*-test and one way ANOVA test) when compared to control. Fermentation time was 7 d. The isolation of peptide components from VI, VII and VIII peptide fractions is shown in Fig. 4, control (CPD) 2·5%. Peptides VI.1, VII.5, and VIII.5 did not flocculate. Lf $ml^{-1}$ values were obtained as the lowest antitoxin dilution leading to flocculation with tetanus toxin

In the basic fraction, the main peptide found to increase toxin production when added to the media was Glycil-L-Histidine, suggesting that peptides are important both for bacterial growth and toxin production (Mueller and Miller 1956). These data were confirmed in the present study using total hydrolysate of the CPD (data not shown). In this case, however, the bacteria grew but showed no significant toxin production. Only the neutral fraction gave reproducible results with CPD; it also gave the best toxin production, confirming previous work by Mueller and Miller (1955) and Stainer (1973). Thus, the neutral fraction of CPD contains different active peptides; these were isolated (Figs 3 and 4) and analysed. Of the peptides obtained after HPLC, only the peaks I, II, VI, VII and VIII showed significant activity above the control. Low peptide concentrations, such as 10 μg, had no effect whereas 100 μg gave maximum stimulation of toxin production (data not shown). Eight of the 13 isolated peptides were sequenced (Fig. 6 and Table 1) and six of them proved to be active.

These active peptides isolated from CPD showed some sequence similarity with the bradykinin-potentiating peptides which also have a high content of proline (Stewart 1979). The primary sequence of several sequenced peptides showed the presence of a Pro-hydrophobic-Pro sequence. This motif is found in several zinc peptidase inhibitors which act as bradykinin potentiators by inhibiting angiotensin-converting enzyme. They were found in the *Bothrops jararaca* venom, casein tryptic digest and in other proteins (Stewart 1979; Maruyama *et al.* 1987; Lebrun *et al.* 1995). As the culture medium contains zinc proteases (Hase and Finkelstein 1993) which include the tetanus toxin light chain, it may be suggested that these peptides could play a role in toxin production by inhibiting its degradation. Another possibility would be the direct action of these peptides as peptide-hormones which stimulate *Cl. tetani* toxin secretion. For example, in the bacterium *Proteus mirabilis*, Bonato *et al.* (1982) found that digested gelatin increased protease secretion 2·5-fold compared to non-digested gelatin.

The search for a more specific and defined medium for production purposes is of great interest in the quest to obtain highly pure antigens. This may not be the case for the preparation of large quantities of toxin for vaccine production because of the high cost of synthetic peptides compared to CPD. However, the use of a totally synthetic medium containing only amino acids and peptides as protein sources for growth and toxin production could be an advantage for production of small quantities of pure toxin used for tetanus toxin detection assays or for high quality antibodies.

**Table 1** Sequences of peptides isolated from the CPD neutral fraction after HPLC purification and their location in different casein chains

| Number | Amino acid sequence | Location | Casein chain |
|---|---|---|---|
| VIa1 | Ile-Pro-Ile-Gln-Tyr-Val | 26–31 | κB-casein bovine |
| VIIb1 | Val-Leu-Gly Pro-Val | 197–201 | β-casein bovine |
| VIId1 | Ala-Val-Pro-Tyr-Pro-Gln | 177–182 | β-casein bovine |
| VIId2 | Asp-Met-Pro-Ile | 184–187 | $\alpha_{s1}$-casein bovine |
| VIIIb1 | Val-Leu-Pro-Val-Pro-Gln | 170–175 | β-casein bovine |
| VIIIb3 | Leu-Val-Tyr-Pro-Phe-Pro | 58–63 | β-casein bovine |
| VIIIc1 | Val-Ala-Pro-Phe-Pro-Glu-Val-Phe | 40–47 | α1-casein bovine |
| VIIId2 | Glu-Met-Pro-Phe-Pro-Lys | 123–128 | β-casein bovine |

## ACKNOWLEDGEMENTS

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) Proc. 95/2960–1, Financiadora de Estudos e Projetos (FINEP) Proc. 66.96.0142–00 and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil. The authors thank Patricia H.C.S. do Couto for technical assistance and Antonia P.M. da Silva for help with the manuscript.

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